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Analysis of LC3-Associated Phagocytosis and Antigen Presentation.

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MHC class I internalization via autophagy proteins

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Summary

Macroautophagy is a ubiquitous degradative pathway involved in innate and adaptive immunity. Its molecular machinery has been described to deliver intracellular and extracellular antigens to MHC class II loading compartment by regulating phagosomes maturation. We recently found that the respective Atg proteins can contribute to MHC class I-restricted antigen presentation to $CD8^+$ T cells by regulating MHC class I surface levels in mouse dendritic cell. Indeed, we found that MHC class I molecules are stabilized on the cell surface of murine antigen presenting cells deficient for core components of the macroautophagy machinery such as Atg5 and Atg7. This stabilization seems to result from defective internalization of MHC class I molecules dependent on adaptor protein kinase 1 (AAK1), involved in clathrin-mediated endocytosis. Moreover, macroautophagy-dependent stabilization of MHC class I molecules leads to enhanced $CD8^+$ T cell priming during influenza A virus infection *in vivo*, resulting in decreased pathology. In this chapter, we describe four experiments to monitor, characterize and quantify the effect of macroautophagy deficiency on MHC class I molecules trafficking and the subsequent $CD8^+$ T cells priming. First, we will show how to monitor MHC class I internalization in lung $CD11c^+$ cells from mice lacking key components of the macroautophagy machinery. Then, we will propose a method to characterize the interaction between either MHC class I or Atg8/LC3 with AAK1. Finally, we will describe how to evaluate the influenza A-specific $CD8^+$ T cell response in mice conditionally depleted for *Atg5* in their DCs compartment. This set of experiments allows to characterize MHC class I internalization with the help of the molecular machinery of macroautophagy

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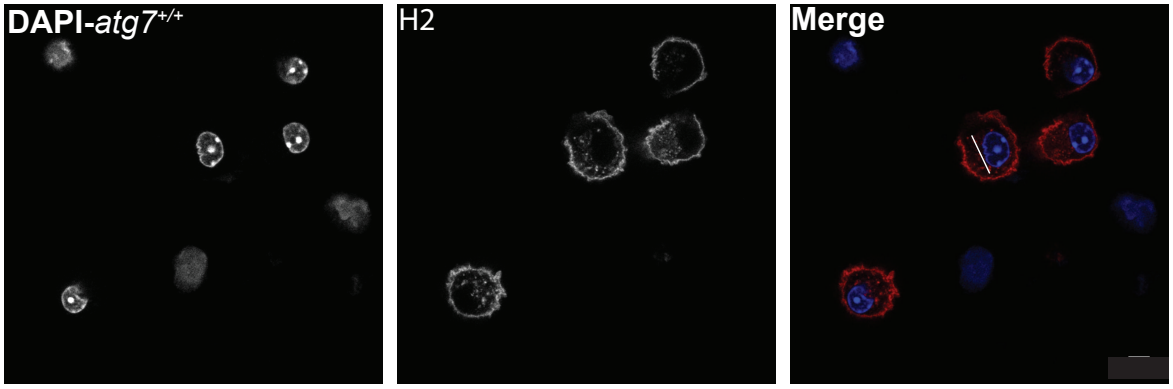
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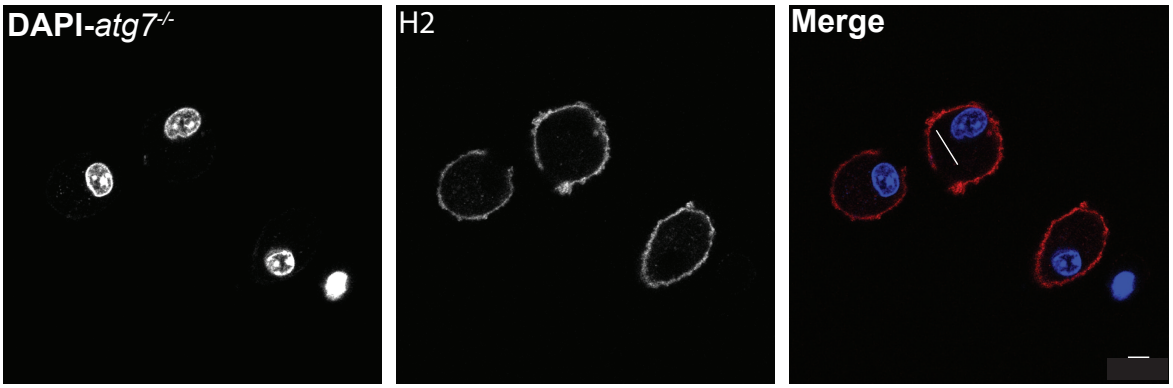
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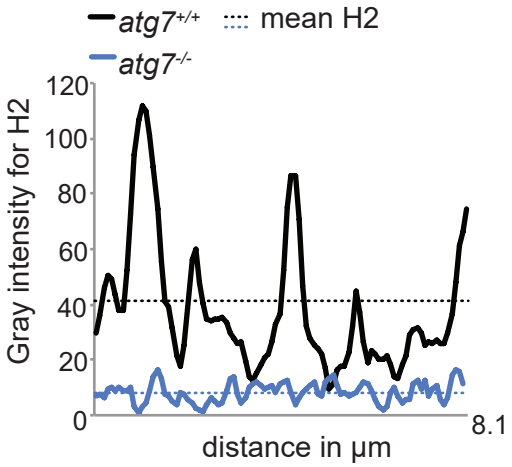
C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mean H2
atg7-/-
Gray intensity for H2
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100
80
60
40
20
0
8.1
distance in µm

1 Introduction

Macroautophagy is a highly conserved degradative pathway among all eukaryotic cells, which mediates delivery of non-functional cytosolic proteins or organelles to the lysosome for degradation. The molecular mechanisms involved during macroautophagy are fairly well known and more than 30 autophagy related proteins (Atg) have been described to play a role to generate a double-membrane vesicle, called autophagosome (1). The microtubule-associated protein light chain LC3 (LC3, mammalian orthologue of yeast Atg8) is by far the best characterized of the Atg proteins and generally considered as the hallmark of the autophagosome. Indeed, LC3 exists in two forms, a cytosolic form (LC3-I) and a lipidated form associated with autophagosomal membranes (LC3-II). LC3 lipidation is under the control of a molecular cascade including the two proteins Atg5 and Atg7 (2). These proteins are frequently targeted to compromise macroautophagy by blocking LC3 lipidation. Macroautophagy has first emerged as an important catabolic process involved during innate immunity, and more recently it appears to play a role also during adaptive immunity. The fact that approximately 20% of the natural major histocompatibility complex (MHC) class II ligands originate from cytosolic and nuclear proteins, including the macroautophagy marker LC3, suggest a possible link between macroautophagy and antigen processing for MHC presentation (3). It has been shown that the macroautophagy machinery assists and facilitates MHC presentation to T cells through different pathways. Its molecular machinery was described to deliver intracellular antigens to MHC class II loading compartments (4), to enhance exogenous antigen delivery for MHC class II presentation (5), but also described to facilitate efficient antigen cross-presentation on MHC class I molecules (6). Macroautophagy plays also an important role in endogenous self-protein processing involved in the negative and positive selection of $CD4^+$ T cell in the thymus (7-8). In addition to the involvement of classical macroautophagy in MHC class II antigen processing, we have shown that exogenous antigen processing for MHC class II presentation can also be regulated via a non-canonical macroautophagy pathway, called LC3-associated phagocytosis or LAP (9). Briefly, in this pathway LC3 is directly coupled to the phagosomal membrane and requires reactive oxygen species production by NADPH oxidase 2 (NOX2), which, depending of the cell type seems to accelerate or attenuate the fusion of phagosomes with the lysosomes (9-11). In case of human macrophages, conventional and plasmacytoid dendritic cells (DCs), the fusion between the phagosome and lysosome is attenuated, resulting in prolonged MHC class II presentation or cargo delivery to toll like receptor (TLR) containing endosomes (9). Compared to the role of macroautophagy during antigen processing for MHC class II presentation, little is known of how this pathway can influence antigen processing for MHC class I presentation. Recently, we demonstrated that the macroautophagy machinery contributes to MHC class I-restricted antigen presentation to $CD8^+$ T cells by regulating MHC class I surface levels in mouse cells leading to enhanced $CD8^+$ T cell priming during influenza A virus infection *in vivo* (12*)*. In fact, mouse dendritic cells with a compromised macroautophagy machinery showed an elevated MHC class I surface expression, which seems to result from a defective internalization of MHC class I molecule dependent on adaptor protein kinase 1 (AAK1). Indeed, AAK1 can interact with the cytosolic form of LC3 but it fails to be recruited to the MHC class I internalization machinery when LC3 lipidation is blocked.

Furthermore, we showed that the impairment of LC3 lipidation by *atg5* or *atg7* gene know-out in murine lung DCs and macrophages, decreases the intracellular pool of internalized MHC class I molecules. Interestingly, in absence of Atg dependent MHC class I internalization, dendritic cells stimulate influenza-specific $CD8^+$ T cell responses more efficiently. This was also associated with better immune control of influenza infection *in vivo,* showing that the macroautophagy machinery orchestrates T cell immunity by attenuating MHC class I surface expression levels (12).

In order to monitor how MHC class I internalization is affected by the macroautophagy machinery, we will describe four methods. First, we will propose two independent but complementary experiments to assess the level of MHC class I internalization in Atg deficient cells. The first method is a flow cytometry-based assay allowing to follow the internalization of antibody-labelled MHC class I molecules over time and compare the rate of internalization between Atg sufficient and deficient cells. The obtained data can be validated by visualizing the internalized MHC class I molecules with a confocal microscopy approach. These two methods allow to assess the impact of a compromised macroautophagy machinery on the internalization of MHC class I molecules, but do not give information about the molecular mechanisms involved. To address this point, we investigated the interaction between either MHC class I or LC3 with the adaptor molecule AAK1. Finally, we describe an assay to show the effect of the impairment of Atg dependent MHC class I internalization on $CD8^+$ T cell response during influenza A virus infection. In this assay we take advantage of an influenza A virus infection model in mice in order to study if the macroautophagy machinery can shape the anti-viral $CD8^+$ T cell response by regulating MHC class I surface levels on antigen presenting cells. These basic experiments allow monitoring of the effects of an impaired macroautophagy machinery on MHC class I internalization and to characterize the implications of such regulation *in vivo*.

2 Materials

2.1 MHC class I internalization by FACS

2.1.1 Mice

C57BL/6 (Janvier), *atg5*$^{flox/flox}$ (kindly provided by Dr. Mizushima, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan) (13) and CD11c-Cre Tg (CD11c$^{cre+/-}$, Jackson).

Conditional knock-out mice for disruption of autophagy in the CD11c^{+} cellular compartment, designated *atg5*$^{-/-}$ DC mice, are generated by crossing *atg5*$^{fl/fl}$ with CD11c$^{cre+/-}$ mice. As littermate control mice, *atg5*$^{fl/fl}$ x CD11c$^{cre-/-}$ (*atg5*$^{+/+}$ DC) mice are used.

2.1.2 Cell culture

Cell culture medium**:** RPMI-1640 supplemented with heat-inactivated (*see* **Note 1**) 10% foetal calf serum (FCS) (R10).

2.1.3 Reagents

1. DNaseI grade II (Roche 7600105) used at a concentration of 50 µg/ml.
2. Hepes 1M (Invitrogen 15630-056) used at a concentration of 25 mM.
3. Collagenase A (Sigma C9891-500MG) used at a concentration of 400 µg/ml.
4. CD11c beads, (Miltenyi 130-052-001) used at a concentration of 100 µl/10^8 cells.
5. Percoll (GE Healthcare 17-0891-01).

2.1.4 Probes

1. Biotin anti-mouse H2-K^{b} (clone AF6-88.5, Biolegend 116504) diluted 1:50 in phosphate buffer saline (PBS).
2. Biotin anti-mouse-H2-D^{b} (clone KH95 Biolegend 111504 diluted) 1:50 in PBS.
3. PE-streptavidin (Biolegend 405203) diluted 1:400 in FACS buffer.
4. Pacific Blue anti-I-A/I- E (clone M5/114.15.2, Biolegend 107620) diluted 1:400 in FACS buffer
5. PE-Cy7 anti-mouse CD11c (clone N418, Biolegend 117318) diluted 1:400 in FACS buffer.
6. Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen L34957) diluted 1:500 in FACS buffer.

2.1.5 Buffers

1. MACS buffer: 1% Bovine serum albumin (BSA), 2mM EDTA in PBS, to be filtered 0.22 µm.
2. FACS buffer: PBS supplemented with 2% FCS and 0.01% sodium azide.

2.2 MHC class I internalization by immunofluorescence

2.2.1 Mice

C57BL/6 (Janvier), *atg5*$^{flox/flox}$ (kindly provided by Dr. Mizushima, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan) (13), *atg7*$^{fl/fl}$ (kindly provided by Dr. Komatsu, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan) (20) and CD11c-Cre Tg (CD11c$^{cre+/-}$, Jackson).

Conditional *knockout* mice for disruption of autophagy in CD11c^{+} compartment, designated *atg5*$^{-/-}$ or *atg7*$^{-/-}$ DC mice, are generated by crossing *atg5*$^{fl/fl}$ or *atg7*$^{flox/flox}$ with CD11c$^{cre+/-}$ mice. As littermate control mice, *atg5*$^{fl/fl}$ x CD11c$^{cre-/-}$ (*atg5*$^{+/+}$ DC) and *atg7*$^{fl/fl}$-CD11c$^{cre-/-}$ (*atg7*$^{+/+}$ DC) mice are used.

2.2.2 Cell culture

1. Cell culture medium**:** RPMI-1640 supplemented with heat-inactivated (*see* **Note 1**) 10% FCS (R10).
2. Multi chamber 8-wells (IBIDI).

2.2.3 Reagents

1. DNaseI grade II (Roche 7600105) used at a concentration of 50 µg/ml.
2. Hepes 1M (Invitrogen 15630-056) used at a concentration of 25 mM.
3. Collagenase A (Sigma C9891-500MG) used at a concentration of 400 µg/ml.
4. CD11c beads (Miltenyi 130-052-001) used at a concentration of 100 µl/10^8 cells.
5. Percoll (GE Healthcare 17-0891-01).
6. Poly-L-Lysine (Sigma) diluted at 1:10 in water.
7. 4% paraformaldehyde solution in PBS (PFA) (Santa-Cruz).
8. Dako fluorescence mounting medium (Dako S3023).

2.2.4 Probes

1. Purified rat anti–mouse CD16/32 (mouse BD Fc block) (BD 553141) diluted at 1:250 in blocking buffer.
2. Biotin anti-mouse H2-K^{b} (clone AF6-88.5, Biolegend 116504) 1:50 in blocking buffer.
3. PE anti-mouse-H2 (clone M1/42, Biolegend 125506) diluted 1:50 in PBS.
4. AlexaFluor®- 555 conjugated goat anti-rat IgG H&L (Invitrogen A21434) diluted 1:500 in blocking buffer.
5. AlexaFluor®- 555 conjugated goat anti-mouse IgG H&L (Invitrogen A21422) diluted at 1:500 in blocking buffer.
6. 4,6-diamidino-2-phenylindole (DAPI) nucleic acid stain, stock solution 5mg/ml. Work solution: 1:10000 in blocking buffer.

2.2.5 Buffers

1. MACS buffer: 1% BSA, 2mM EDTA in PBS, filtered 0.22 µm.
2. FACS buffer: PBS supplemented with 2% FCS and 0.01% sodium azide.
3. Coated solution: Poly-L-Lysine (Sigma) diluted at 1:10 in sterile water.
4. Permeabilization solution: 0.1% Triton X-100 in PBS.
5. Blocking buffer: PBS supplemented with 1% of bovine serum albumin (BSA).

2.3 Immunoprecipitation for MHC class I and LC3, followed by Western blotting for AAK1

2.3.1 Mice

C57BL/6 (Janvier), *atg5*$^{flox/flox}$ (kindly provided by Dr. Mizushima, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan) (13), *atg7*$^{fl/fl}$ (kindly provided by Dr. Komatsu, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan) (20) and CD11c-Cre Tg (CD11c$^{cre+/-}$, Jackson).

Conditional knock-out mice for disruption of autophagy in CD11c^{+} compartment, designated *atg5*$^{-/-}$ or *atg7*$^{-/-}$ DC mice, are generated by crossing *atg5*$^{fl/fl}$ or *atg7*$^{flox/flox}$ with CD11c$^{cre+/-}$ mice. As littermate control mice, *atg5*$^{fl/fl}$ x CD11c$^{cre-/-}$ (*atg5*$^{+/+}$ DC) and *atg7*$^{fl/fl}$-CD11c$^{cre-/-}$ (*atg7*$^{+/+}$ DC) mice are used.

2.3.2 Cell culture

1. BM-DCs medium filtered sterile RPMI-1640 supplemented with penicillin/streptomycin (50 U/ml, 15070-063, lifescience), 50 µM β-mercaptoethanol (Bio-Rad, 161-0710) (*see* **Note 2**), 20ng/ml GM-CSF and 20% heat inactivated FCS (*see* **Note 3**).

2.3.3 Reagents

1. ACK lysis buffer (0.15 M NH4 Cl, 1 mM KHCO3, and 0.1 mM EDTA, pH 7.2), (Invitrogen A10492-01).
2. Protein A beads (GE Healthcare 17-0780-01).
3. BCA protein assay kit (Thermo Scientific 23225).
4. Complete protease inhibitor cocktail tablets (Roche 4693116001).
5. ECL Amersham ECL Detection Reagents (GE Healthcare).
6. Acrylamide/Bis-acrylamide: 30%, ratio 29:1 (Bio-Rad 161-0156).
7. TEMED (N,N,N,N-Tetramethylethylenediamine) (Bio-Rad 161-0800).
8. Protein Marker, dual color (Bio-Rad 161-0374).
9. Amersham Hybond 0.45 µm PVDF membrane (GE Healthcare 10600023).
10. Blotting paper: 100M ROLLE DC PAPIER 3MMCHR 100MM (Whatman) (Fischer Scientific W3788R)

2.3.4 Probes and cytokine

1. Normal rabbit anti serum (NRS) (Jackson 011-000-120) diluted 1:20 in cold IP lysis buffer.
2. Rabbit anti-mouse H2-K^{b} (Exon-8) (a gift from Dr. Jack Bennink, Bethesda, MD) used 5 µl.
3. Biotin anti-mouse H2-K^{b} (clone AF6-88.5, Biolegend 116504) diluted 1:1000 in in PBS-T-1% BSA.
4. Rabbit anti-LC3 (MBL PM036) used 5 µl for IP and diluted 1:1000 in PBS-T-1% skimmed milk for WB.
5. Rabbit anti-AAK1 (Abcam ab77082) diluted 1:1000 in in PBS-T-1% BSA.
6. Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (HRP) (Jackson) diluted 1:50000 in in PBS-T-1% BSA.
7. Streptavidin HRP (Mabtech 3310-9) diluted 1:1000 in in PBS/T-1% BSA.
8. Human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Biolegend 576302), stock solution 0.20 mg/ml, used at 20 ng/ml.
9. Pacific Blue anti-I-A/I-E (clone M5/114.15.2,Biolegend 107620) diluted 1:400 in FACS buffer.

10. PE-Cy7 anti-mouse CD11c (clone N418, Biolegend 117318) diluted 1:400 in FACS buffer.
11. Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen L34957) diluted 1:500 in FACS buffer.

2.3.5 Buffers

1. IP Lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40.
2. SDS-PAGE Loading Buffer: 50 ml dH_2O, 16 ml 1.5 M Tris–HCl pH 6.8, 15 ml glycerol, 5g SDS, few flakes of Bromphenol blue, store aliquots at −20°C, add 1 % of β-mercaptoethanol to the sample buffer before use.
3. Resolving gel buffer: 1.5 M Tris–HCl pH 8.8, store at room temperature (Bio-Rad 161-0798).
4. Stacking gel buffer: 0.5 M Tris–HCl pH 6.8, store at room temperature (Bio-Rad 161-0799).
5. Initiator (APS): 10% ammonium persulfate solution (APS, Sigma A3678-25) in dH_2O, prepare just prior to use or store aliquots at -20°C.
6. SDS-PAGE Running buffer: dilute 10× Tris/Glycine/SDS (TGS, Bio-Rad 161-0772) with dH_2O to obtain 1× TGS final: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.
7. Transfer buffer: dilute 20× NuPAGE® Transfer Buffer (20X) (life technologies NP0006-1) with dH_2O to obtain 1× TG final (25 mM Tris, 92 mM glycine, pH 8.3) and add 20% of methanol.
8. PBS/T: 0.1% Tween-20 in PBS.
9. Blocking buffer: 5% skimmed milk powder in PBS/T.

2.4 Influenza infection of $CD11c^{cre+/-}$ x $Atg5^{fl/fl}$ mice and read-out for $CD8^+$ T cell responses in the lung

2.4.1 Mice

C57BL/6 (Janvier), *atg5*$^{flox/flox}$ (kindly provided by Dr. Mizushima, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan. (13) and CD11c-Cre Tg ($CD11c^{cre+/-}$, Jackson).

Conditional knock-out mice for disruption of autophagy in the $CD11c^+$ cellular compartment, designated *atg5*$^{-/-}$ DC mice, are generated by crossing *atg5*$^{fl/fl}$ x $CD11c^{cre+/-}$ mice. As littermate control mice, *atg5*$^{fl/fl}$ x $CD11c^{cre-/-}$ (*atg5*$^{+/+}$ DC) mice are used.

2.4.2 Cell culture

1. Cell culture medium**:** RPMI-1640 supplemented with heat-inactivated (*see* **Note 1**) 10% FCS (R10).

2.4.3 Reagents

1. DNaseI grade II (Roche 7600105) used at a concentration of 50 µg/ml.
2. Hepes 1M (Invitrogen 15630-056) used at a concentration of 25 mM.
3. Collagenase A (Sigma C9891-500MG) used at a concentration of 400 µg/ml.
4. CD11c beads (Miltenyi 130-052-001) used at a concentration of 100 µl/10^8 cells.
5. Percoll (GE Healthcare 17-0891-01).
6. Brefeldin A (Sigma B5936) used at concentration of 10 µg/ml.
7. BD Cytofix/Cytoperm Fixation/Permeabilization Kit (554714).

2.4.4 Probes

1. APC-Cy7 anti-mouse CD45 (clone 30-F11, BD 557659 diluted) 1:50 in FACS buffer.
3. Pacific Blue anti-mouse CD4 (clone GK1.5, Biolegend 100428) diluted 1:500 in FACS buffer.
4. APC anti-mouse CD8 (clone 53-6.7, Biolegend 100712) diluted 1:125 in FACS buffer.
5. Anti-mouse CD28 (BD 553294) used at 4 µg/ml.
6. Anti-mouse CD3 (BD 553057) used at 10 µg/ml.
7. PE-anti-IFN-γ, XMG1.2 (BD 554412) diluted 1:50 in 1x Perm/wash solution (BD kit).
8 Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen L34957) diluted 1:500 in FACS buffer.

2.4.5 Virus and peptide

1. Influenza A/PR8 virus (H1N1) Charles River.
2. Peptides $NP_{1366-374}$, $HA_{211-225}$, $NP_{2311-325}$, and NY-ESO-$1_{157-170}$ were synthesized by GL Biochem (Shanghai) at a purity >90 %, used at 20 µg/ml.

2.4.6 Buffers

1. FACS buffer PBS supplemented with 2% FCS and 0.01% sodium azide.

3. Methods

3.1 MHC class I internalization by FACS

3.1.1 Lung digestion

1. Euthanize *atg5*$^{-/-}$ or *atg7*$^{-/-}$ DC mice and their littermates with CO_2 inhalation (*see* **Note 4**).
2. Perfuse immediately the lungs with 10 ml PBS from the left ventricle of the heart with 18G needle.
3. Remove the lung in 5 ml in R10 and keep on ice.
4. Transfer the lungs in a 1.5 ml collection tube for mechanical pre-digestion by dissecting it with scissors.
5. Transfer the obtained lung preparation in a 6-well plate (one lung/well). To easier collect the lung suspension, cut the tip of the P1000.
6. Wash the collection tube with 1 ml of R10 and transfer the remaining 4 ml of R10 into the well.
7. Add the following digestion cocktail: 400 µg/ml Collagenase A, 50 µg/ml DNase I, 25 mM Hepes and incubate for 45 minutes at 37°C on shaker.
8. Incubate for additional 5 minutes at 37°C with 10 mM EDTA to stop the reaction.
9. Put a 40 µm strainer on top of a 50 ml falcon tube and equilibrate the strainer with 10 ml cold PBS. Keep samples on ice. Press the tissue through the strainer using the piston of the 2,5 / 5ml syringe.
10. Wash the cell strainer with 10 ml cold PBS, Centrifuge at 500 x g for 10 minutes and aspirate the supernatant.

11. Prepare 15 ml falcon tube with 70% Percoll and overlay it with the cells previously resuspended in 4 ml of 40% Percoll (*see* **Note 5**).
12. Add 1ml of RPMI-1640 on top. Spin 2590 rpm with an acceleration of 4 and deceleration of 1 during 30 minutes at 4°C.
13. Carefully collect the tubes out from the centrifuge to not disrupt the gradient. Remove the top ring (CD45 negative fraction, or collect it if needed) and collect the intermediate ring (CD45 positive fraction) in 50 ml falcon tube.
14. Wash with 40 ml cold PBS, 500 x g for 10 minutes (*see* **Note 6**).
15. Resuspend the pellet in 5 ml PBS per 1ml of R10 based on the further use and count the cells. Average yield from one mouse: 1-4 x 10^7 CD45-positive cells total.

3.1.2 $CD11c^+$ MACS separation

1. Resuspend cells isolated from lung (see Subheading 3.1.1) at a concentration of 2.5 x 10^8 cells/ml with cold MACS buffer and add 100 µl CD11c beads per 10^8 cells at 4°C.
2. After 20 minutes, wash in cold MACS Buffer, 500 x g for 5 minutes, and then resuspend 2 x 10^8 cells/ml in cold MACS buffer.
3. Proceed with positive magnetic cell separation in the autoMACS cell separator (Miltenyi Biotec). Choose positive selection “posseld” program (*see* **Note 7**).
4. Take the positive fraction; centrifuge at 400 × g at 4°C for 10 minutes.
5. Carefully remove the supernatant and resuspended cells in 100 µl of PBS and then count cells. These cells are enriched for $CD11c^+$ cells with purity of 72-80%.

3.1.3 Internalization assay

1. For each time point, transfer 2 x 10^5 CD11c sorted dendritic cells (see Subheading 3.1.1) in a FACS tube and wash with 2 ml PBS once (500 x g for 5 minutes).
2. Cells are stained in FACS tube with either anti-H2-D^b or anti-H2-K^b biotinylated antibodies, or their respective isotypes, diluted at 1:50 in FACS buffer for 30 minutes at 4°C (*see* **Note 8**).
3. Wash cells 2 times in 2 ml PBS, 500 x g for 5 minutes, and resuspend in 200 µl R10. Keep cells at 4°C or incubate at 37°C, for 10, 30, 60 and 90 minutes (*see* **Note 9**).
4. After the incubation time, wash cells with 2 ml PBS, 500 x g for 5 minutes and perform surface staining at 4°C for 30 minutes with FACS buffer with following antibody mix:
    - 1:400 PE-coupled streptavidin
    - 1:400 PE-Cy7-anti-CD11c
    - 1:400 PB- anti-I-A/I-E
    - 1:500 Aqua (see Subheading 2.1.4)
5. Wash cells with 2 ml PBS 500 x g for 5 minutes, resuspend in 100 µl FACS buffer and acquire samples at the BD FACSCanto-II.
6. Analyse the data in two steps.

(a) Subtract the mean fluorescence intensity (MFI) of the PE fluorescence of the isotype from the MFI of the MHC class I.

(b) Evaluate the decrease of MFI intensity compared to the MFI of control DCs incubated at 4°C set as a reference at 100%.

3.2 MHC class I internalization by immune fluorescence microscopy

3.2.1 Staining of MHC class I

1. 8-well chamber slides are coated by adding 50 µl of poly-L-Lysine and incubated for 10 minutes at room temperature (RT) (*see* **Note 10).** Then, they are gently washed three times with sterile PBS. Prepare some extra wells, which will be used for control conditions and stainings (*see* **Note 11**).
2. Wild type or *atg7*-deficient lung CD11c-positive cells are selected as described in the subheading 3.1.1 and are plated into pre-coated wells at the density of 2 $x10^5$ cells/ well.
3. After overnight cultured at 37°C and 5% CO_2, the cells are spun down at 500 rpm during 3 minutes and then gently and carefully washed with sterile PBS 1 time (*see* **Note 12 and 13**).
4. Directly add Fc blocker 2.4G2Fcc III/II diluted at 1:250 in PBS and incubates cells during 30 minutes at 4°C.
5. The cells are directly stained with 20 µl of the primary antibody against MHC-class I for 30 minutes at 4°C (dilution 1:50) and then transferred to 37°C for 1h. MHC-class I can be stained by using an anti-H2-K^b or anti-H2 antibody.
6. Labelled cells are spun down at 500 rpm for 3 minutes and gently washed 1 time with PBS.
7. After a centrifugation step at 500 rpm for 3 minutes the cells are fixed with 4% paraformaldehyde during 20 minutes at room temperature.
8. Fixed cells are carefully washed three times with PBS and then permeabilized with 0.1% Triton X-100 for 5 minutes at RT, afterwards the cells are washed with PBS (*see* **Note 14**).
9. Add the blocking buffer (PBS-BSA 1%) and incubate during 1hour at RT.
10. AlexaFluor 555 is used as a secondary antibody (*see* **Note 15**). The secondary antibody (1:500) and DAPI nucleic acid stain (1:10 0000) are diluted into the blocking buffer and then 20 µl are added to each well.
12. After 1 hour of incubation at RT in the dark the cells are washed three times with PBS.
13. Remove any trace of PBS and then carefully detached the plastic chamber from the slide (*see* **Note 16**).
14. Add a drop of the mounting medium Dako, and then on top a coverslip of 1.5 mm thickness. Each coverslip is carefully pressed down and dried at room temperature in the dark (*see* **Note 17**). Slides could be stored for several months at 4°C in the dark.

3.1.2 Immune fluorescence analysis

1. Cells were analysed with an upright confocal laser-scanning microscope (SP8, Leica), using a 63x, NA 1.4 oil immersion objective (*see* **Note 18**). The excitation is performed at 405 nm to elicit the DAPI

fluorescence (emission max 470 nm, blue fluorescence) and an excitation at 543 nm elicits the AlexaFluor®-555 (emission max 580 nm, red fluorescence). Images are acquired using Leica software (Leica), then analysed and assembled using ImageJ software.

2. To appreciate and quantify the internalization of MHC class I, the intensity across trajectories through vesicular MHC class I is recorded by using the Image J software. The obtained data are represented by profile plot of MHC class I fluorescence intensity. The number of MHC class I vesicle can be quantified by hand or using an available plugging from ImageJ.

3. Figure 1 shows the internalization of MHC class I assessed by immunofluorescence. $CD11c^+$ cells from lungs were treated and analysed following the procedure described above. Figure 1A shows the localization of MHC class I in Atg sufficient $CD11c^+$ cells. Figure B shows the localization of MHC class I in *atg7*$^{-/-}$ $CD11c^+$ cells. Figure 1C shows the profile plot of MHC class I intensity in the cytoplasm.

3.3 Immunoprecipitation for MHC class I and LC3, followed by Western blotting for AAK1

3.3.1 Isolation of DCs from the bone marrow

1. Euthanize *atg5*$^{-/-}$ and their littermates with CO_2 inhalation, place the mouse in dorsal recumbency on a clean dissection board and spray legs with 70% ethanol.

2. With help of scissors and forceps, remove skin and muscle overlaying femur and tibia.

3. Remove the bones by cutting between femur and hip joint and remove the remaining muscle with the help of a paper tissue. Then, transfer into falcon tubes with phosphate buffered saline (PBS) on ice (*see* **Note 19**).

4. Cut the femur from tibia, cut open distally and introduce a syringe with a 30G x 12 mm needle into the bone marrow channel to flush out the bone marrow with sterile PBS.

5. Wash with PBS 1500 rpm for 10 minutes.

6. Force cell suspension through a 70 µm strainer and centrifuge 1500 rpm for 5 minutes.

7. Lyse erythrocytes by adding 1 ml ACK Lysis buffer (See Subheading 2.3.5) and incubate 3 minutes at RT, then wash with PBS at 1500 rpm for 5 minutes (*see* **Note 20**).

8. Plate cells at 3 x 10^6 /ml BM-DC Medium (See Subheading 2.3.2) in 100 x 15 mm non-tissue coated petri dishes (*see* **Note 21**).

9. Perform a full medium change every second day by collecting the cells, spinnning them down and resuspending them in fresh BM-DC medium (*see* **Note 22**). Place them back into their original dish.

10. On day 8-10 for mature DCs (*see* **Note 23**) remove all the culture medium from each plate (*see* **Note 24**).

11. Wash the plate with 2-3 ml PBS, trypsinize cells with 2 ml trypsin for 5 minutes at 37°C, inactivate the trypsin with 8 ml medium and collect it.

12. Add 5 ml of ice-cold PBS and mechanically detach the cells from the plate by cell scraper.

13. Collect, centrifuge (1500 rpm for 5 minutes) and count them. From one mouse, 5-8 x10^7 total cells can be isolated (*see* **Note 25**).

3.3.2 Preparation stock IP beads

1. For a final volume of 2 ml beads, add 40 ml of distilled water (dH_2O) to 400 mg of lyophilized protein A (*see* **Note 26**).
2. Incubate 1 hour at RT under rotation.
3. Spin down at 300 x g for 3 minutes or allow beads to settle. Decant off supernatant. Wash 3 times with 50 mM Tris Buffer pH7.0.
4. Resuspend in equal medium volume of 50 mM Tris buffer pH7.0 + 20% ethanol (EtOH) for long-term storage at 4°C (if we have 2 ml of beads, add 2 ml of EtOH/Tris buffer).

3.3.3 IP beads washing

1. For one IP experiment, transfer 300 µl of beads to an Eppendorf tube.
2. Wash 3 times with 500 µl PBS, spin at 1500 x g for 1 minute.
3. Resuspend them in 300 µl cold lysis IP buffer (See Subheading 2.3.5).

3.3.4 Immunoprecipitation for MHC I and LC3

1. The whole cell lysis and IP procedures has to be done at 4°C.
2. Grow BM-DCs from *atg5*$^{-/-}$ and *atg5*$^{+/+}$ as described in (See Subheading 3.1.1). After 10 days post differentiation, collect and count BM-DCs. For MHC class I and LC3 pull-down the use of at least $2x10^7$ cells is recommended (*see* **Note 27**).
3. Wash the cells 3 times with PBS and transfer the cells to a 1.5 ml collection tube.
4. Resuspend with cold IP Lysis buffer ($1x10^6$ cells/50 µl) containing appropriate protease inhibitors (see Subheading 2.3.5). Incubate at 4°C for 30 minutes, then vortex briefly (up to 10 seconds).
5. Centrifuge tubes at 13000 x g for 10 minutes at 4°C to remove nuclei and transfer the supernatant to another tube.
6. Measure protein concentration by BCA to be sure to have the same amount of proteins between *atg5*$^{-/-}$ *and atg5*$^{+/+}$ lysates.
7. To pre-clear the cell lysate from protein that non-specifically bind the beads, add 50 µL of washed beads (see Subheading 3.3.3)(*see* **Note 28**) to the cell lysate and incubate them for 1hour at 4°C with rotation.
8. Spin at 1500 x g for 10 minutes to pull down beads and harvest supernatant. Be careful to keep sample free of beads. Keep 50 µl of pre-cleared lysate as control for the WB.
9. Divide your cell lysate in two collection tubes.
10. Add primary antibody or its isotype/normal serum. For instance, 5 µl anti-EXON 8 or 2 µl anti-LC3 and the corresponding amount of NRS diluted in IP Lysis buffer (See Subheading 2.3.4) are added to cell lysate and mix well.
11. Immunoprecipate your sample overnight at 4°C under rotation (*see* **Note 29**).
12.The next day, add 50 µl of beads resuspended in the cold Lysis buffer. Mix well and incubate with gentle rotation at 4°C for 1 hour.

13. Spin beads down at 2500 xg for 1 minute, remove the supernatant and add 1:20 cold Lysis buffer (*see* **Note 30**). Repeat this step 3-5 times.
14. After washes, denature the beads in 20 µl SDS-PAGE loading buffer (see Subheading 2.3.5), 3 minutes at 95°C.
15. Centrifuge at 12000 x g for 10 minutes at 4°C and collect the supernatant (*see* **Note 31**).
16. Load 20 µl of the sample per lane in a 1.5 mm thick SDS-polyacrylamide gel for electrophoresis.

3.3.5 Western blotting for AAK1

1. All procedures have to be carried out at RT unless otherwise specified.
2. Prepare 7% and 12.5% gel mixture for analysing association between MHC class I and AAK1 and LC3 and AAK1, respectively. See recipe in Table 1:

Table 1. Recipe for resolving gel

	7%	12.5%
Resolving Buffer	2.5 ml	2.5 ml
Acrylamide mixture	2.33 ml	4.2 ml
dH2O	5 ml	3.15 ml
10% SDS	100 µl	100 µl
TEMED	15 µl	15 µl
APS	75 µl	75 µl

Cast 7.5 ml of gel mixture within a 7.25 cm × 10 cm × 1.5 mm gel cassette (Bio-Rad MINIPROTEAN III system). Gently overlay with water and wait until the gel mixture is polymerized (*see* **Note 32**).
3. To prepare the stacking gel mix 750 µl of stacking gel buffer, 325 µl of acrylamide mixture, 1.5 ml of water, 50 µl of 10% SDS, 18.75 µl of APS, and 3.75 µl of TEMED. Insert a 10- or 15-well gel comb immediately without introducing air bubbles.
4. Remove the water from the gel cassette by simply flipping the system and cast 2 ml of stacking gel as prepared above.
5. Perform electrophoresis at constant amperage with SDS-PAGE running buffer. Start with 60 mA until the sample has entered the gel and then continue at 90 mA till the dye front has reached the bottom of the gel.
6. Cut PVDF membrane to the size of the gel and immerse it in 100% methanol for 5 minutes. Rinse once with transfer buffer for 5 minutes.
7. Immediately after electrophoresis, separate the glass gel plates with the help of a spatula or similar tool.
8. Perform semi-dry transfer with Mini Trans-Blot Electrophoretic Transfer Cell, using standard procedure with transfer buffer at 15 mA for 1 hour (*see* **Note 33**).
9. Block the membrane with blocking solution at least 45 minutes at RT or overnight at 4°C under agitation.
10. Cut the membrane according to the molecular weight (MW) of the interested proteins and incubated it whit the corresponding primary antibody diluted 1:1000 in PBS/T-1% BSA for 3 hours at RT or overnight at

4°C under rotation. Rabbit anti-AAK1 (MW 94 kDa), biotin anti-mouse H2-K^b (MW 46 kDa), and rabbit anti-LC3 Ab (MW 18-15 kDa) (See Subheading 2.3.4)

11. Wash with PBS/T once for 15 minutes and three times for 5 minutes.

12. Add the secondary antibody, goat anti-rabbit IgG HRP-conjugate (dilution 1:50000 in PBS/T-1% BSA) or Streptavidin HRP (diluted 1:1000 in in PBS/T-1% BSA (See Subheading 2.3.4) and incubate for 1 hour at RT.

13.Wash with PBS/T: once for 15 minutes and four times for 5 minutes.

14. Reveal using the ECL kit following the manufacturer's instructions.

3.4 Influenza infection of CD11c-cre x *atg5*$^{fl/fl}$ mice and read-out for CD8$^+$ T cell responses in the lung

3.4.1 Influenza virus infection in vivo.

1. Thaw virus on ice.

2. Prepare 10 HAU virus suspension and keep it on ice.

3. Anesthetized mice using isoflurane (*see* **Note 34**). After the mouse is deeply anaesthetized, confirmed by the absence of reflex on the footpad, hold the mouse by its ears and let it lean along your hand.

4. Gradually inject 25 µl (*see* **Note 35**) of virus suspension into the nostrils (divide the volume in the two nostrils) with the help of micropipette with filtered tip. Adjust the rate of release to allow the mouse to inhale without forming bubbles. Inoculate control mice with 25 µl of PBS.

5. Hold the mouse in the hanging position for another couple of minutes until its breathing gradually returns normal.

6. Weight the mouse. Weight loss is used to monitor the development of the infection.

3.4.2 Evaluation of CD8$^+$ T cell response by IFN-γ quantification by FACS

1. Label 4 sterile FACS tubes with cap the day before (*see* **Note 36**) for each condition.

2. Incubate lung single cell suspensions obtained after Percoll gradient centrifugation (*see* Subheading 3.1.1) in R10 at 37°C for 5 hours in the presence of 4 µg/ml anti-mouse CD28 (*see* Subheading 2.4.4) and 20 µg/ml influenza specific peptides. Use approximately 1x10^6 cells in 250 µl per staining.

3. Add 250 µl CD28/influenza peptide solution in RPMI without serum and incubate for 1 hour at 37°C. Setup 3 Controls: (a) 10 µg/ml anti-mouse CD3 (*see* Subheading 2.4.4); (b) PMA 1 µg/ml and ionomycin 1 µM final concentration; (c) No peptide/no anti-CD28.

4. Add 50µl/ tube of Brefeldin A solution (10 µg/ml in RPMI) and incubate for additional 4 hours at 37°C.

5. Wash cells once with at least 2 ml of cold PBS (*see* **Note 37**), spin at 500 x g for 5 minutes, discard the supernatant.

6. Use 50 µl /tube of the following antibody mix:

- 1:500 Pacific Blue anti-mouse CD4,
- 1:125 APC anti-mouse CD8,
- 1:50 APC-Cy7 anti-mouse CD45,

- 1:500 Aqua Live/Dead diluted in FACS buffer (*see* Subheading 2.4.4).

Incubate 20 minutes on ice.

7. Wash with 2 ml cold PBS, spin at 500 x g for 5 minutes 4°C.

8. Resuspend cell pellet in 250 µl Cytofix/Cytoperm (BD Kit) and incubate for 20 minutes on ice, then wash with cold PBS.

9. Resuspend cell pellet in FACS Buffer, store o/n in the fridge.

10. Wash cells with 1ml of 1x Perm/Wash Buffer (BD kit, 1:10 dilution in dH_2O). Spin at 400 x g for 5 minutes, discard supernatant.

11. Add 50µl of PE-anti-mouse IFN-γ antibody (See Subheading 2.4.4) diluted 1:50 in 1x Perm/Wash solution (BD kit). Incubate for 25-30 minutes.

12. Wash with 1x Perm/Wash.

13. Resuspend in FACS buffer and acquire the sample the same day using a cytometer.

4. Notes

1. FCS batches need to be heat inactivated before use in culture to denature complement that may be present in serum. Place thawed bottle of FCS in 56°C water bath for 40 minutes. Be sure to mix contents manually by swirling regularly to ensure even temperature distribution and reduce precipitation of the FCS. FCS being added to RPMI should be passed through a 0.2 µm filter to ensure sterility and reduce precipitates within Culture Media.

2. β-Mercaptoethanol is used as reducing agent because cells at 20% O_2 produce ROS that leads to oxidation of antioxidant pools. To avoid oxidative stress, most cultured cells amplify antioxidant defence, however certain cell types are unable to do so and thus β-mercaptoethanol is added to aid in maintaining a reducing environment. It is recommended to add β-mercaptoethanol freshly.

3. FCS is one component that profoundly influences BM-DC generation. Indeed, it has been shown that even different FCS lots or batches of the same product could drastically affect BM-DC generation. Therefore, it is highly recommended to make test cultures for a side-by-side comparison with different FCS (15).

4. Avoid cervical dislocation because it results in rapid death but is traumatic. This technique damages the trachea and cervical region and confounds the results by causing haemorrhage into various tissues and contaminating the lung tissue.

5. Carefully first prepare 90% Percoll with 10X PBS in order to have further dilutions that are isotonic.

6. After centrifugation, make sure Percoll has been completely washed out because it might be toxic for the cells.

7. Filter the cells before going to the autoMACS because cells could aggregate. Moreover, to keep the cells at 4°C put the collecting tubes in a refrigerator rack.

8. To be more consistent and be sure that all cells get exactly the same staining, you could also stain them in one tube and divide them after washing for the different time points.

9. The incubation time has to finish at the same moment to achieve better consistency. Start with the 90 minutes incubation, keeping the other tubes at 4°C. Put the next tube after 30 minutes (time point 60 minutes) and so on.

10. Poly-L-Lysine is a charged enhancer used for coating the wells to promote better cells adhesion. The poly-L-Lysine is diluted at 1:10 in water and has to be freshly made every time. Some cell types are able to digest the poly-L-Lysine, in this case it is preferable to use the poly-D-Lysine.

11. For correct interpretation of the result, the following controls should be included:

- One well should be incubated without any primary or secondary antibody but only with the blocking buffer to determine the cells auto fluorescence.
- One slide should be incubated only with the secondary antibody to assess the background due to the secondary antibody. Background of the secondary antibody should be low.

In case of multiple stainings, include slides to perform single labelling of cells and check the leak-through fluorescent in the adjacent channel. For instance, AlexaFluor®-555 will be analysed with the green channel and no significant signal should be detected.

12. The overnight culture is an important step to give time to the cells to nicely adhere and spread the cytoplasm on the well surface.

13. CD11c positive cells do not adhere very strongly to the bottom of the well, in order to limit the loss of cells, every single step before the fixation must be preceded by a centrifugation at 500 rpm for 3 minutes. The liquid (medium, PBS…) has to be gently and carefully removed with a vacuum suction flask.

14. From the fixation step forward, cells could be handled outside a sterile biosafety hood on a laboratory bench. Use a vacuum suction flask to change solutions and exchange the plastic tip of the suction devise between different solutions and conditions.

15. Secondary antibody coupled with red fluorochromes are preferred to green ones due to the high level of cellular auto-fluorescence in the green channel.

16. The pastic chambers are carefully detached from the slide and with the help of a scalpel remove any traces of glue before adding the mounting medium.

17. Dako mounting medium contains an anti-fading agent, which retards the fading of fluorescence and allows the analysis of the slides long after mounting. During the overnight incubation time in the dark the mounting medium will polymerize and it will not be necessary to seal the coverslips with nail polish.

18. The slide could also be analysed with an inverted confocal laser-scanning microscope having the right lens and laser.

19. It is possible to keep bones at 4°C for 1-2 days in PBS. Cells are not affected, but it might be a bit harder to flush the bones out, since they become softer over time in solution.

20. Do not incubate cells for long periods of time with ACK lysis buffer because it is harmful to the cells.

21. Be careful with respect to the concentrations of your cells. DCs love cell-to-cell contact. Therefore, they do not mind dense conditions. Also, after 3 days of culture, most non-myeloid cells die off leaving extra space for the DCs. On the other hand, too dense conditions, like 5 x 10^6/ml and above, would lead to spontaneous maturation.

22. A complete change to fresh medium is not necessary and a half-old-half-new protocol works well too.

23. After 3 days of culture, it is already possible to detect around 20% of $CD11c^+$ MHC II^{high} cells. The percentage increases over time up to 80% at day 8-10. Before using the cells, always confirm purity and maturation status of DC cultures by FACS staining: 1:400 PE-Cy7-anti-CD11c, 1:400 PB-anti-I-A/I-E, and 1:500 Aqua (See Subheading 2.3.4) in FACS buffer for 30 minutes. 80% of $CD11c^+$ MHC II^{high} DCs should be achieved.

24. For a correct interpretation of the data, be aware that BM-DC populations are heterogeneous. Indeed, it has been shown that the $CD11c^+MHCII^+$ fraction of GM-CSF cultures comprises at least two cell types that by ontogenetic and gene expression criteria correspond to monocyte-derived macrophages and CDP-derived DCs (16).

25. It is possible to freeze the cells at any time during their maturation process. We recommend to freeze the cells down right after the isolation. Resuspend them in freezing medium at a concentration of 2 x 10^6 per ml and freeze the cells in cryo-tubes (1ml per tube).

26. Protein G is often considered the more universal IgG Binding Protein compared to protein A, but different species, and isotypes of species, do vary in their binding to these proteins, *see* Table 2. Therefore, choose wisely with respect to protein A versus G usage.

Table 2. Protein A/G Affinities for Polyclonal Sera

Ab Isotype	Affinity
Human	Protein A or G
Horse	Protein G
Cow	Protein G
Pig	Protein A or G
Sheep	Protein G (weakly)
Goat	Protein G (weakly)
Rabbit	Protein A or G
Chicken	Protein G (weakly)
Hamster	Protein G (weakly)
Guinea Pig	Protein A
Rat	Protein G (weakly)
Mouse	Protein A or G (both weakly)

27. The minimum input for the co-immunoprecipitation is 1 g of protein per condition. Usually, for 3 × 10^6 cells, 250–300 µg of total protein is expected.

28. The pre-clearing step is optional and depends on the nonspecific binding due to charges, and/or contact of hydrophobic surfaces.

29. In case of high levels of unspecific binding, shorten the incubation step of the primary antibody to 45 minutes.

30. Other possible changes to reduce the unspecific binding is to adjust washing stringency and washing steps. Move toward a higher stringency buffer (*i.e.* use up to 1% Tween-20, a nonionic detergent, up to 0.2% SDS, an anionic charged detergent, or up to 1 M NaCl) and increase the number of washing steps.

31. Be careful not to take beads in order to avoid carry-over of contamination.

32. Assemble the system and before pouring the resolving buffer, fill the system with dH_2O and leave it for 5 minutes. Sometimes the system is not perfectly sealed and it could leak out. If there is no leakage, remove the water by just flicking the system, otherwise reassemble the system.

33. If you are not confident in your transfer, it is possible to incubate the PVDF membrane with Ponceau S (0.1 % (w/v) in 5% acetic acid) staining, a reversible staining to check protein bands. After less than 5 minutes of incubation, it will be possible to visualise the bands as a red staining that can be remove simply by washing with dH_2O.

34. The type of anaesthesia during intranasal instillation is an important variable that could have a significant impact on its efficiency for delivery of inocula to the lungs. In fact, mice can swallow and the gastrointestinal tract is heavily influenced by level of anaesthesia and the volume of the inoculum. Several studies have shown that the infection is significantly more efficient when the injection is performed under isofluorane inhaled anaesthesia in comparison to the parenteral-administered ketamine/xylazine. It has been speculated that this could be because the irregular Cheyne-Stokes type respiratory pattern that is typically observed following inhalation of anaesthesia causes transient hypoxia which results in deeper inhalation of larger volumes of inoculum per breath, facilitating more efficient delivery of the material to the lower respiratory tract (17). Accordingly, increasing the depth of isoflurane anaesthesia (230 µl/dm^3) improved the infectivity of the large-volume inoculum, probably because of suppression of swallow and sneeze reflexes (18). In contrast, mice that receive injectable anaesthesia breathe in a more regular and more shallow pattern, resulting in a more coating of the upper respiratory tract surface with the inoculum that leads to an inefficiently delivery of the inoculum to the lower tract.

35. Another important variable for the intranasal inoculation of anaesthesia is the volume. It is relatively well established that intranasal instillation for delivery to the upper respiratory tract requires a low administration volume (≤10 µl). However, no real consensus has been reached with respect to dose volume for delivery to the lower respiratory tract. It has been shown that a brief respiratory distress occurs in mice that have received intranasal instillation volumes ≥50 µl (19).

36. For easier and faster handling, it is possible to perform the assay in U-bottom 96-well plate. Perform washing steps with 200 µl, centrifugation step at 2200 rpm for 2 minutes. Volumes: stimulation in 100 µl, add 20 µl Brefeldin A, extracellular and IFN-γ staining in 20 µl.

38. Make sure the medium is washed out. RPMI is known to inhibit Aqua staining.

Figure 1. Influence of the macroautophagy pathway on the internalization of MHC class I molecules in murine lung CD11c$^+$ cells, analyzed by confocal microscopy. Lung CD11c$^+$ cells from wild-type mice (*atg7*$^{+/+}$) **(a)** and mice with a deficiency in the macroautophagy machinery (*atg7*$^{-/-}$) **(b)** were stained for MHC class I with anti-H2 antibody (red channel). Nuclear DNA was counterstained with DAPI (blue channel). Merged images, corresponding to the compilation of the two individual staining, clearly show how the cytoplasmatic pool of MHC class I molecules after internalization is compromised in *atg7* deficient CD11c positive cells. Scale bar indicates 5 µm. **(c)** The fluorescence intensity of vesicular MHC class I was recorded along the trajectories, as depicted in the merged images of b).

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